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Neutrophil and recombinant myeloperoxidase as antigens in ANCA positive systemic vasculitis

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SUMMARY

Myeloperoxidase (MPO) is one of the major autoantigens recognized by anti-neutrophil cytoplasm antibodies. The association of this antigen with specific disease entities requires that there is a source of pure antigen present in large quantities. Further delineation of the molecular mechanisms involved in the antigen–antibody interaction requires the ability to manipulate the molecule. The expression of recombinant MPO in Chinese hamster ovary cells has produced a source of pure protein, suitable for molecular studies. We have shown that this protein is an antigen recognized by 95% of anti-MPO antibodies from patients with systemic vasculitis. This recombinant molecule will be of use in providing an additional specific solid-phase assay for these antibodies and further forms of this protein which mirror the antigenicity of native MPO more exactly may replace chemically purified antigen. It will also be of great value in studies examining the epitopes recognized by anti-MPO antibodies and in studies of immunoregulation and T cell activation.

Keywords myeloperoxidase recombinant ANCA systemic vasculitis rapidly progressive glomerulonephritis autoimmunity

INTRODUCTION

Anti-neutrophil cytoplasm antibodies (ANCA) are autoantibodies associated with several diseases characterized by a small vessel vasculitis, in particular Wegener's granulomatosis (WG) [1], microscopic polyangiitis (polyarteritis, MPA) [2] and a renal limited form of this disease known as idiopathic rapidly progressive glomerulonephritis [2]. Two patterns of antibody binding have been defined by their indirect immunofluorescence appearance on normal human polymorphonuclear leucocytes. The first is a granular cytoplasmic (classical) pattern (C-ANCA), the second a predominantly perinuclear pattern (P-ANCA) [3]. The majority of C-ANCA sera recognize proteinase-3 (PR3) [4], a 29-kD lysosomal antigen whilst the predominant antigen recognized by P-ANCA sera is myeloperoxidase (MPO) [2].

Myeloperoxidase is a lysosomal enzyme with two symmetrical heavy chains (58 kD), and two light chains (16 kD). The molecule can exist as one heavy and one light chain (hemiperoxidase) and is enzymically active in this form. Recently recombinant myeloperoxidase (rMPO) has been expressed in Chinese hamster ovary (CHO) cells [5] and is secreted into the culture medium as an 84-kD single chain protein equivalent to a molecule of hemiperoxidase with the promolecule retained at the *N*-terminus. Large quantities of the pure molecule can thus be produced in the laboratory. Although there is a minor difference in the glycosylation, probably representing a failure of terminal mannose processing, the haem-binding domains are identical [6] and the recombinant molecule is enzymically active.

In this study we compared the antigenicity of native MPO and rMPO using a panel of sera from patients with vasculitis which we have previously shown to contain autoantibodies which bound to native MPO. In addition, because other studies from our laboratory indicated that commercial MPO (Calbiochem, Nottingham, UK) contained trace amounts of lactoferrin [7], another ANCA antigen which is recognized by certain vasculitic sera, we used affinity purified (lactoferrin-free) MPO, as well as commercial MPO, in the comparison studies with the recombinant protein. This contamination of MPO by lactoferrin is well recognized and it is not easy to be sure that MPO produced chemically is in fact completely pure. The small amount of lactoferrin present in the preparations is sufficient to give strong antigenic responses in ELISA and in immunization studies [7,8], and this was again demonstrated in our study.

Our aim was to determine whether rMPO could be used as an alternative for native MPO as a solid phase ligand in ELISA

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in vitro and therefore also whether it might be effective as an extracorporeal immunoabsorbent *in vivo*, since increasing evidence points to the pathogenic importance of anti-MPO antibodies and the large quantities of MPO necessary for such a therapeutic strategy would be available using recombinant DNA technology. We wished to know if rMPO would be of use in other areas of research, namely epitope mapping and studies of immunoregulation.

MATERIALS AND METHODS

Characterization of patients with anti-MPO associated vasculitis

Anti-MPO sera. Sera containing anti-MPO autoantibodies were collected from patients with ANCA positive vasculitis. These were characterized by ANCA ELISA, as well as indirect immunofluorescence (IIF) and a subsequent specific MPO ELISA using a commercial MPO preparation (see below). Sera from 59 patients that bound to MPO were selected for the study. All aliquots were stored at −20°C.

ANCA ELISA. An acid extract from human neutrophils [9] was coated for 3 h at 37°C to polystyrene ELISA plates (Dynatec, Billingshurst, UK), and non-specific binding blocked with PBS containing 2% gelatin (PBS-Gel). The plates were then washed three times with PBS containing 0·1% Tween-20 (PBST) and sera were incubated 1 : 20 in PBS containing 1% gelatin and 0·1% Tween-20 (PBS-Gel-Tw). Then the plates were washed and incubated with a mouse monoclonal anti-human IgG (Oxoid M06014; Basingstoke, UK) diluted 1 : 200 in PBS-Gel-Tw. After washing, binding of the mouse IgG was detected with an alkaline phosphatase-conjugated polyclonal anti-mouse IgG (Sigma A-5153; Poole, UK) diluted 1 : 1000 in PBS-Gel-Tw and, after addition of substrate (Sigma 104), the plates were read at 405 nm. Controls included PBS and normal sera, and all samples were simultaneously assayed on antigen-free wells [9]. The binding of a test serum was expressed as a percentage of the binding of a reference positive serum and was considered positive if it had a titre greater than 25% (NR < 13%). All volumes were 100 μl, times of incubations were 1 h and all incubations were performed at 37°C.

Indirect immunofluorescence. All sera were screened for cytoplasmic staining on normal ethanol permeabilized human neutrophils according to previously described techniques [1] and the pattern of immunofluorescence recorded as either C-ANCA or P-ANCA. Only sera positive for ANCA and MPO in the ELISA assays and for ANCA on immunofluorescence were used in this study.

Purification of native MPO

Preparation of semipurified native MPO for use in affinity chromatography. A modification of the method of Andrews & Krinsky was used [10]. Briefly, granulocytes from whole blood and buffy coats were separated on a methylcellulose/Hypaque gradient and lysed in 0·5% cetyltrimethylammonium bromide (CTAB) overnight. Cell debris was pelleted and the supernatant was removed. Ammonium sulphate was added (310 mg/ml) to produce a 50% saturated solution and the precipitate removed. A further 100 mg/ml ammonium sulphate was added and the MPO pelleted by centrifugation. After resuspending in 100 mM phosphate buffer pH 7·5, the MPO was further purified on a cation exchange column (Mono S, Pharmacia, Uppsala, Sweden). Fractions were eluted with a NaCl gradient and tested for peroxidase activity and iron content as described. Fractions containing MPO were passed down a Superose 12 size separation column (Pharmacia) and the resulting MPO was free of all known contaminants except trace amounts of lactoferrin when screened for other specificities by ELISA. Four milligrams of this MPO were used to produce an MPO-affinity column by coupling the MPO to cyanogen bromide activated Sepharose 4B (Pharmacia). This column was used to produce affinity purified anti-MPO antibodies (see below).

Preparation of immunoabsorbent column containing immobilized affinity purified anti-MPO antibodies. Serum from a patient with high titres of anti-MPO antibodies and without other autoantibodies as detected by the panel of ELISA assays described below was applied undiluted to a Protein G column (Pharmacia). The IgG from 50 ml of serum was eluted from the column with 0·1 M glycine/HCl pH 2·8 and, after neutralization with NaOH, was run down the semi-purified MPO affinity column. Bound anti-MPO antibody was then eluted as before and activity tested on an MPO ELISA. The affinity purified antibodies were dialysed against coupling buffer (0·1 M $NaHCO_3$, 0·5 M NaCl pH 8·3) and then attached to cyanogen bromide activated Sepharose 4B to form an affinity antibody column. This column was used to produce highly purified MPO. Four affinity columns were produced, one from a serum that subsequently was shown to have markedly reduced binding to rMPO relative to purified MPO. Both rMPO and the purified preparation of native MPO were passed over these columns as a further way of determining whether there were differences in binding of the two preparations.

Preparation of highly purified native MPO. One milligram of MPO diluted in PBS was passed over the column; the bound fraction was eluted off with 0·1 M glycine/HCl pH 2·8 and the pH restored to neutral with NaOH. This MPO retained enzymic activity and when tested in ELISA with specific MoAb, was free of other contaminating proteins, in particular lactoferrin. Only small quantities of purified MPO could be prepared in this way.

Purification of rMPO

Recombinant MPO was purified from culture supernatant of CHO cells using a two step chromatography procedure [5]. In brief the culture medium was passed through a Q-Sepharose column in phosphate buffer and the flow-through fraction was loaded onto a carboxymethyl-Sepharose fast-flow column. Recombinant MPO was eluted with a NaCl gradient and all fractions tested for enzyme activity in an MPO-capture ELISA. All steps were carried out at 4°C. Fractions containing the rMPO were checked for absorbance characteristics, enzymic activity and were run on SDS–PAGE to confirm purity.

MPO activity assay

The enzymic activity of the various antigens was assayed in order to confirm that the amounts coated onto each plate were of equivalent activity. Briefly, the peroxidase activity in each was measured using 3,3,5,5-tetramethylbenzidine as substrate for the hypochlorous acid produced by the action of MPO on H_2O_2 in PBS [12]. Absorbance was measured at 655 nm.

ELISAs using different MPO preparations

MPO ELISA. MPO (Calbiochem) was coated onto Dynatec ELISA plates at a concentration of 0·1 units/ml overnight at

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4°C. The activity of this enzyme as assayed by Calbiochem defines 1 unit as the amount of enzyme that will decompose 1 μM of H_2O_2 per min at 25°C, pH 6·0. The specific activity of this preparation is stated to be between 60 and 200 units/mg.

Thereafter the sequence of steps was as described for the ANCA ELISA above except that sera were incubated 1 : 50, no mouse anti-human IgG antibody step was required and detection of binding was with an alkaline phosphatase-conjugated goat anti-human IgG (Jackson Immunoresearch 109-055-088; Westgrove, PA) 1 : 3000 in PBS-Gel-Tw [11]. A monoclonal mouse anti-MPO antibody (Dako M748) was used as the positive control at a concentration of 1 : 500 and detected with a goat anti-mouse alkaline phosphatase-conjugated antibody (Sigma A5153) used at a concentration of 1 : 1000. The binding of the test sera were expressed as a percentage of the binding of the mouse anti-MPO MoAb which was arbitrarily assigned a value of 100% for each assay.

Native MPO (highly purified) ELISA. MPO purified as above was coated onto plates at 0·1 units/ml. All other steps were as above. Binding was expressed as a percentage of the mouse anti-MPO MoAb control.

MPO (recombinant) ELISA. Recombinant MPO was coated onto Dynatec plates at 0·1 units/ml (0·1 μg/ml). Recombinant MPO has a specific activity of around 100 units/mg. All other steps were as above. Binding was expressed as a percentage of the mouse anti-MPO MoAb control. In separate assays, seven sera were repetitively assayed on both native and rMPO and binding compared to the mouse monoclonal anti-MPO antibody.

Detection of contaminants in the different MPO ligands

To detect possible contaminating autoantigens in these MPO preparations, each MPO ELISA was probed with the following antibodies: a mouse MoAb to elastase (Dako M752) diluted 1 : 500, a rabbit polyclonal antibody to lactoferrin diluted 1 : 1000 (Dako A186), a sheep polyclonal antibody to cathepsin G diluted 1 : 1000 (ICN 647321) and a MoAb to lactoferrin used as neat culture supernatant, produced in our laboratory.

ELISAs for other vasculitis autoantibodies

To determine whether the sera contained autoantibodies to other vasculitis autoantigens the following ELISAs for antibodies to lactoferrin, lysozyme, cathepsin G, proteinase-3 and elastase were performed. A DNA ELISA was also performed because some of the sera were known to have anti-nuclear antibodies and because myeloperoxidase is believed to associate with DNA due to the high PI of MPO (PI = 11) and low PI of DNA. Preliminary studies were performed to optimize assay conditions using reference positive sera. Criteria for positivity were established for each assay.

Lactoferrin ELISA. Briefly, lactoferrin (Sigma L0520) obtained from human milk was coated at a concentration of 4 μg/ml onto Dynatec plates and the sera were coated at a 1 : 50 dilution in PBS-Gel-Tw. After a 1 h incubation the plate was developed as above.

Lysozyme ELISA. Lysozyme (Sigma L6394) from human milk was coated at a concentration of 2 μg/ml and the assay performed as above.

Cathepsin G ELISA. Cathepsin G (Sigma C4428) from human leucocytes was coated at a concentration of 0·5 μg/ml and the assay performed as above.

Proteinase-3 and elastase ELISA. These assays were performed in our laboratories by Dr M.H. Zhaó using PR3 and an elastase-enriched preparation from normal human neutrophils prepared according to the method of Kao *et al.* [13]. Antigen was coated at a concentration of 0·2 μg/ml and 2·0 μg/ml, respectively and the assays performed as above.

DNA ELISA. Highly polymerized calf thymus DNA (Sigma D1501) was coated onto plates at a concentration of 40 μg/ml. The plates had been precoated with 0·2% protamine sulphate [14]. After blocking with 0·5% gelatin for 1 h sera were coated at a 1 : 100 dilution and binding detected as above. No attempt was made to remove any single stranded DNA.

SDS polyacrylamide gel electrophoresis and Western blotting

All SDS–PAGE was performed using a 'Mighty small' gel apparatus (Hoeffer Scientific Instruments, Newcastle under Lyme, UK), with a 12% resolving gel and a 4% stacking gel [15]. All samples were reduced with 2-mercaptoethanol (2-ME, Sigma M6250) and the gel was run at 15 mA whilst the proteins were in the stack and at 25 mA when they reached the resolving gel. Bromophenol blue was used as a marker dye (Sigma B8026). Appropriate molecular weight markers were run in one lane (Gibco BRL).

Immunoprecipitation studies

To confirm specificity for rMPO, sera from six patients and affinity purified antibodies from one patient with equivalent binding to both native and rMPO and from one patient with reduced binding to rMPO were tested for their ability to immunoprecipitate rMPO. rMPO (200 μg) was dialysed into 0·1 M sodium phosphate buffer pH 7·2 at a concentration of 1 mg/ml. Biotinamidocaproate-*N*-hydroxy-sulphosuccinimide ester (4 μl: BAC-SulfoNHS, Sigma B4908) (10 mg in 60 μl DMSO) was added to 200 μl rMPO and the mixture agitated for 2 h [16]. Excess BAC-SulphoNHS was removed by passing the mixture over a Sephadex-G25M column with PBS. Biotinylated ligand (10 μl) was then added to 90 μl 10 mM 4-hydroxyazobenzene-2-carboxylic acid (HABA, Sigma H5126) and compared with a PBS control in order to estimate the number of moles of biotin coupled per mole of rMPO.

Serum (10 μl) from each of the eight patients was added to 10 μl of biotinylated rMPO and incubated overnight at 4°C. Normal sera and omission of antigen were used as controls. Washed Pansorbin cells (100 μl: Calbiochem 507861) were then added and agitated gently for 2–3 h. The cells were centrifuged and excess supernatant discarded. After washing three times with PBS, the antigen–antibody complexes were eluted from the beads with reducing Laemmli sample buffer and applied to a standard SDS–PAGE, run at 25 mA. The protein was then transferred at 0·5 A onto nitrocellulose membrane in 3-cyclohexylamino-1-propanesulphonic acid buffer, pH 11·5 (CAPS, Sigma C2632) for 40 min, after which the membrane was blocked with 10 mM Tris, 150 mM NaCl, 0·1% Tween-20 and 2% Marvel (TBSTM) and then probed with avidin-alkaline phosphatase 1 μg/ml (Sigma A2527) for 1 h in TBSTM. After washing, the membrane was developed with substrate (0·066 μg/ml 5-bromo, 4-chloro,3-indolylphosphate (Sigma B8503) and 0·132 μg/ml nitro-blue tetrazolium (Sigma N6876) in 100 mM Tris pH 9·5 containing 100 mM NaCl and 5 mM $MgCl_2$). The membrane could take several hours to develop due to the small amounts of protein involved.

Table 1. Sera with altered binding to recombinant MPO

Patient	Commercial	Recombinant	Purified
Non-binding			
4	63	< 5	61
6	66	< 5	54
9	61	9	60
Reduced binding			
2	64	30	67
3	87	19	67
5	90	55	99
7	59	24	57
8	70	22	80
11	82	22	102
16	67	35	60
18	105	68	107
20	69	31	65
21	129	32	133
26	94	28	73
39	58	35	54

Top: three sera that do not bind to recombinant MPO despite binding to the native preparation. Below: 12 sera that have reduced binding to the recombinant molecule relative to the native preparation. Figures represent the binding of each serum to the three MPO preparations expressed as a percentage of the binding of the mouse MoAb, used as the positive control throughout.

RESULTS

ANCA ELISA and indirect immunofluorescence

All 59 sera were positive in the ANCA assay with titres greater than 25% (NR < 13%). All sera produced positive indirect immunofluorescence on normal human neutrophils, 43 having a P-ANCA pattern, 14 having a C-ANCA pattern and two having an indeterminate pattern.

MPO antigens

Recombinant MPO, from Dr N. Moguilevsky in Nivelles, was tested for enzyme activity using the *o*-dianisidine assay [17] and had a specific activity of 100 units/milligram. Ratio of absorbance at 430 nm/280 nm was 0·66, whilst that for native MPO was 0·75. The reduced value for rMPO is well reported and felt to represent the effect of the promolecule [5]. SDS–PAGE confirmed a single band running at 84 kD for rMPO [5], and confirmed bands at 72 kD, 58 kD and 16 kD for the native protein. A coating concentration for the three antigens of 0·1 units/ml, determined from assessment of their peroxidase activities and optical densities at 280 nm was used throughout.

MPO ELISAs

All sera were screened in all three MPO assays. The results shown in Table 1 and Fig. 1 are derived from several repeated sets of assays. All 59 sera bound to the commercial and the affinity purified MPO. However 3/59 sera consistently did not recognize the recombinant MPO preparation and 12/59 had a binding reduced by more than 35% to this preparation. In a separate experiment, this reduction in binding was unaffected by doubling the coating concentration of the recombinant antigen on the plate. Three of 59 sera appeared to show increased binding to the recombinant preparation and 7/59 sera showed increased binding to the commercial preparation. The 12 samples with reduced binding in Table 1 and Fig. 1 represent those samples that had consistently reduced binding in all assays. In repetitive studies, six sera that recognized rMPO equally with the native preparation and one serum with reduced binding to rMPO demonstrated consistent and reproducible binding on many occasions.

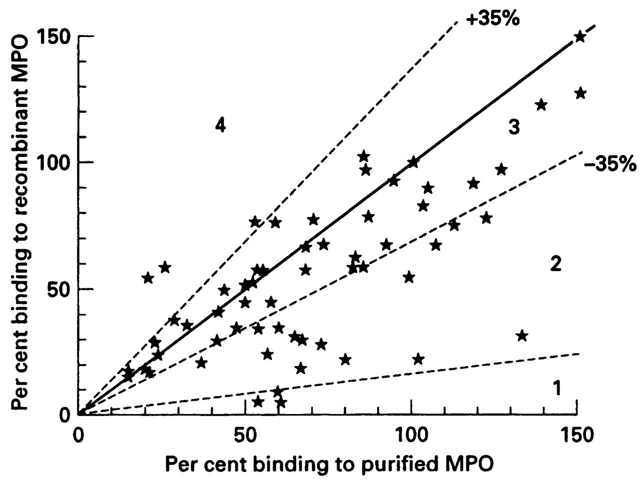

Fig. 1. The four binding patterns to recombinant MPO are shown. Group 1, three sera that did not recognize rMPO; Group 2, 12 sera with reduced binding to rMPO; Group 3, 41 sera with equivalent binding; Group 4, three sera with increased binding to rMPO. ——, Equivalent binding as determined by the mouse MoAb.

The MoAb to elastase and the sheep polyclonal antibody to cathepsin G showed no binding to any of the MPO preparations. The polyclonal antibody to lactoferrin bound very strongly to commercial MPO but did not bind to the other two preparations. The monoclonal antilactoferrin tissue culture supernatant only bound very weakly to the commercial MPO preparation whilst binding very strongly to human lactoferrin. This suggests that the amount of lactoferrin in the batch of commercial MPO used was low (see Table 2).

ELISAs for antibodies to lactoferrin, elastase, cathepsin G, PR3, lysozyme and DNA

In addition to binding to MPO, two sera contained antibodies to PR3, 12 sera were positive in the lactoferrin assay and 10 were positive in the assay using the elastase-enriched preparation. No sera recognized either lysozyme or cathepsin G. Six sera had antibodies against DNA. The sera with increased binding to the commercial preparation of MPO all had antibodies to lactoferrin. Not all sera positive in the lactoferrin assay had increased binding in the commercial MPO assay, and this was thought to be a reflection of antibody titre. Those with a low level of anti-lactoferrin antibodies did not give a strong enough signal in the commercial MPO ELISA for it to be recognized above that from binding to MPO.

Studies with immunosorbent columns containing immobilized affinity purified anti-MPO antibodies

Equal amounts of both rMPO and affinity purified MPO were passed over a column containing affinity-purified antibodies from one of the 12 patients with markedly reduced binding to the recombinant preparation (binding reduced by 70%). After

Table 2. Screening of different MPO preparations for the presence of contaminating antigens

	Monoclonal antibodies			Polyclonal antibodies	
	Mouse anti-MPO	Mouse anti-LF	Mouse anti-elastase	Rabbit anti-LF	Sheep anti-Cathepsin G
Commercial MPO	+	±	−	+	−
Purified MPO	+	−	−	−	−
Recombinant MPO	+	−	−	−	−

Screening for contaminating antigens revealed that there was detectable lactoferrin in the commercial preparation, which was not seen in the other two preparations. This lactoferrin gave weak reactivity with the MoAb but reacted strongly with the rabbit polyclonal anti-lactoferrin antiserum. No other contaminants were detected.

passage of antigen the columns were eluted with 0·1 M glycine/HCl pH 2·8 and the unbound and eluted fractions tested for MPO activity. With affinity-purified MPO, all activity was in the eluate whereas with recombinant MPO very little activity was found in the eluate with the main activity being in the unbound fraction. When the same amount of rMPO was passed over another column with an equivalent amount of affinity antibody from a patient whose serum recognized the recombinant preparation as well as the native preparation, all the MPO activity was present in the eluted fraction. These results confirmed that the serum with reduced binding to the recombinant preparation did have antibodies with specificity for MPO.

Immunoprecipitation studies

Sera from seven patients with good binding in the rMPO ELISA were able to immunoprecipitate biotinylated rMPO as an 84-kD band (see Fig. 2). However, the serum from the patient with reduced binding to rMPO was not demonstrated to precipitate rMPO in this system and normal control sera also did not precipitate any rMPO. Detection required prolonged incubation in substrate, reflecting the small quantity of protein precipitated and available for recognition in such blotting studies.

DISCUSSION

Human neutrophil MPO is one of the main autoantigens recognized by circulating autoantibodies present in patients with small vessel systemic vasculitis [2] and there is growing evidence that these anti-MPO antibodies might be important in pathogenesis. However, studies to investigate the role of anti-MPO antibodies are hampered by difficulties in preparing adequate quantities of pure enzyme. Currently MPO is obtained from large numbers of neutrophils using biochemical techniques, but the quantity produced is often small. The problem is greater if supplies of cells are limited by the availability of normal blood donors. MPO from leukaemic cells might be a suitable alternative, but there is a risk that MPO from leukaemic cells might be expressed aberrantly [18]. A further difficulty arises since increasingly it is being recognized that most preparations of MPO may contain trace amounts of other lysosomal enzymes, in particular lactoferrin, which may act as autoantigens in their own right. Other as yet unknown antigens may also be present. The preparation of MPO by recombinant DNA technology should obviate these difficulties and provide a consistent and reproducible source of antigen. This can then be used to characterize the antigen–antibody interactions more closely without the problems of contamination. In addition, with the advent of more specific therapies such as extracorporeal plasma perfusion through immunoabsorption devices, the availability of large quantities of pure recombinant human

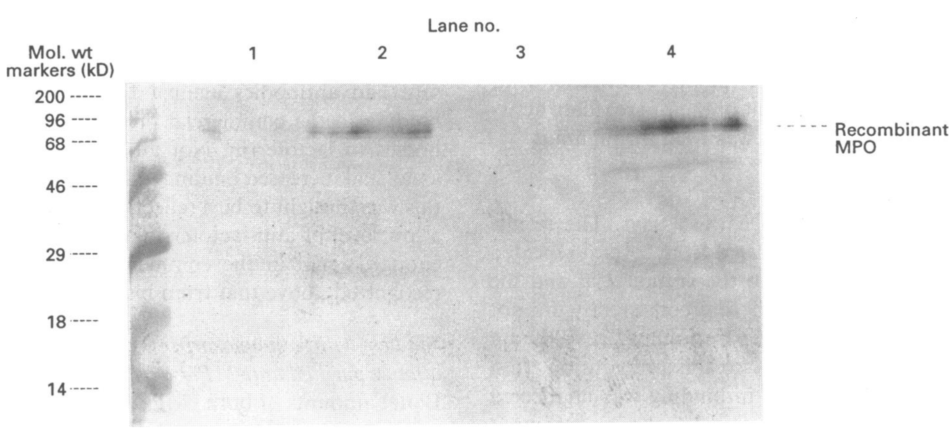

Fig. 2. Immunoprecipitation of rMPO. Lane 1, control serum with rMPO and protein A; lane 2, patient's serum with rMPO and protein A; lane 3, patient's serum with protein A alone; lane 4, patient's affinity purified anti-MPO antibodies with rMPO and protein A.

MPO might allow such a procedure to be carried out using the rMPO as the specific immunoabsorbent. We have therefore initiated studies of rMPO with these objectives in mind.

We have shown here that, after several repeated assays, the recombinant protein is an antigen recognized by 56/59 (95%) of the sera that bind to native preparations. The binding was equivalent in 41/59 sera as judged by results in the MPO ELISAs. Variations in the binding of the 15/59 sera (3/59 increased binding, 12/59 decreased binding) and the lack of binding to rMPO for 3/59 sera might be explained by molecular differences in the recombinant molecule. The three rMPO negative sera were negative on the other antigen ELISAs. By using an affinity antibody column from one of the patients with markedly reduced binding to purify native MPO, we were able to confirm that these sera do indeed have specificity for native MPO whilst failing to recognize rMPO. Immunoprecipitation studies have confirmed the specificity for rMPO for all seven sera used that recognized rMPO well in the ELISA assay. The recombinant protein will therefore be useful in further studies of the molecular mechanisms involved in these diseases.

We have shown further that the pattern of immunofluorescence is a poor marker of antigen specificity. Only two of 14 C-ANCA positive sera had specificity for PR3, whilst all had anti-MPO antibodies. Patterns of immunofluorescence should not be used as definitive markers of specific antigens or diseases. Further, we have again demonstrated the problems in obtaining purified antigens using chemical methods alone. An affinity purification step using an antibody column was necessary to remove all traces of lactoferrin from the commercial preparation, a material which is widely used in studies with these antibodies.

The recombinant protein is not identical to native MPO in several respects. Firstly it is secreted into the culture medium as an 84-kD single polypeptide with no cleavage event to give rise to the heavy and light chain seen in the native hemiperoxidase molecule. This retained six amino acid link peptide could alter the surface configuration in this region of the molecule. Secondly it does not associate with another hemiperoxidase molecule to give the full myeloperoxidase molecule usually present in mature neutrophils. Thirdly, it retains the 108 amino acid, 14-kD pro-molecule and this may well fold over to cover part of the mature protein and prevent dimerization. Fourthly the glycosylation is slightly different with the recombinant protein having a slightly higher mannose content (7·7% v 4·6%) thought to represent a failure of terminal mannose clipping [5]. As one of the glycosylation sites is in the groove between the two hemiperoxidase molecules, the altered glycosylation might be one explanation as to why the two recombinant hemiperoxidase molecules do not come together. However, the lack of processing into light and heavy chains as a prerequisite step for dimerization has also been suggested. It is enzymically active and initial studies suggest that the active site is identical in the two species. The three negative sera may recognize a common epitope that is altered by one or more of these structural differences in the molecule. It is clear that at least one epitope is missing from the recombinant molecule.

With the availability of the recombinant molecule there is now an alternative and consistent source of antigen. However, 5% of sera positive for ANCA will give a false negative result in a subsequent assay to determine antigen specificity for MPO when the recombinant protein in its current form is used. Studies are now in progress to determine whether further manipulation of the construction of the molecule will enable recognition by the remaining 5% of sera and improve its potential as a solid phase antigen. The molecule will be of great use in studies using site-directed mutagenesis and deletion mutants to examine the epitopes recognized by these human autoantibodies and to further establish their fine specificity.

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